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EXPRESSION OF RECOMBINANT GLYCOPROTEINS WITH COMPLETE N-GLYCOSYLATION SITE OCCUPANCY FROM CHO CELLS

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ABSTRACT

Glycosylation of glycoproteins effect immunogenicity, solubility and stability of the protein. Recombinant glycoproteins from mammalian cell culture systems are expressed with heterogeneity in glycosylation. This review describes the methodology from obtaining recombinant glycoproteins without any glycosylation heterogeneity and with total N glycosylation sequon occupancy from CHO cells. The review describes the addition of a glycosylphosphatidylinositol anchor signal sequence C terminal of the recombinant glycoprotein results in complete N-glycosylation site occupancy from CHO mammalian expression system.

KEYWORDS: CHO Expression System, Glycoforms, Recombinant Erythropoietin, Thy-1

INTRODUCTION

In eukaryotic cells the presence of oligosaccharides on a majority of cell surface and secreted glycoproteins are involved in intracellular targeting of glycoproteins, antigenicity, protein solubility and stability [1-3]. Alterations in cell surface oligosaccharides are associated with various pathological conditions such as rheumatoid arthritis [4]. Glycoproteins such as monoclonal antibodies, receptor–IgG fusion proteins, hormones, growth factors and therapeutic enzymes are important biotherapeutics mostly from Chinese hamster ovary (CHO) mammalian cells [5,6].

Glycosylation is of two types N- linked glycosylation at the Asn in the N-glycosylation sequon Asn-X-Ser/Thr and; O-linked glycosylation where oligosaccharides are attached to Ser residues/Thr residues [1]. It has been observed that 10-30% of potential N-glycosylation sites remain unoccupied giving rise to variable site occupancy [7]. The cellular oligosaccharide processing enzymes and the protein primary structure specify protein and site specific glycosylation [8].

GLYCOPROTEIN BIOSYNTHESIS

N-glycosylation of glycoproteins in the endoplamic reticulum is a co-translational process [2, 9]. The transfer of the consensus oligosaccharide structure ($Glc_3Man_9GlcNAc_2$) occurs from a dolichol linked pyrophosphate donor to the Asn residue of the Asn-X-Ser/Thr by the enzyme oligosaccharyl transferase [2, 9]). Modifications or processing of the oligosaccharide occurs in the ER by α1, 2 glucosidase I (Gluc 1) which removes the terminal glucose followed by the action of α1, 2 glucosidase II which removes the remaining 2 glucose molecules [9]. Mannosidase I in the ER removes at least 1 mannose residue before the newly synthesized glycoprotein is transported into the Golgi apparatus for further processing to produce hybrid and complex oligosaccharides by the enzymes N- cetylglucosaminyltransferase 1(GnT1), N-acetylglucos-aminyltransferase II (GnT II) and N-acetylglucosaminyltransferase III (GnTIII) (11). Each of these individual enzymatic reactions may not go to completion giving rise to various glycoforms (**Figure 1**).

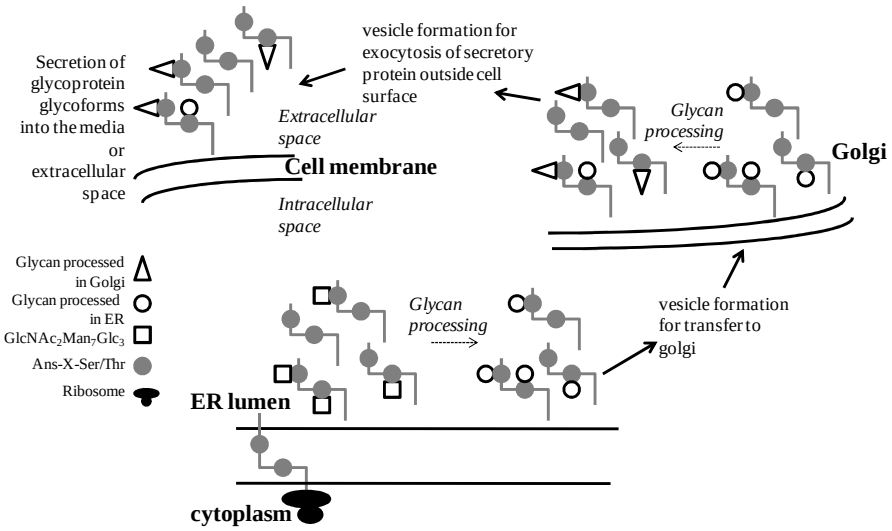

Figure 1: Schematic Representation of Glycoform Formation from CHO Cells

RECOMBINANT GLYCOPROTEIN FROM MAMMALIAN EXPRESSION SYSTEMS

Recombinant glycoproteins from CHO cells have oligosaccharides comprised of oligomannose, hybrid and complex oligosaccharides (9, 10). Recombinant glycoproteins from different cell lines have glycoproteins with cell specific glycosylation. Similarly oligosaccahrides of secreted Thy-1 from CHO-K1 cells were predominatly neutral while from COS -7 cells were both neutral and sialylayed in equal proportion [11,12]. Recombinant secreted glycoprotein expression in mammalian cell lines has almost always been observed with variable glycosylation site occupancy [9, 10-14].

Secretion of Recombinant Glycoproteins from Mammalian Cells

The expression of single recombinant domains ending in a Cys residue involved in disulphide bridge formation can be obtained by the addition of a peptide chain at either end of the domain [9, 11, 15]. Secretion of recombinant soluble Thy-1 was only achieved by the addition of the tripeptide GGS to the terminal Cys 111 residue [11]. Similarly in the expression of recombinant T cell receptor ab domain the addition of 10-15 amino acid residues at the C-terminus was necessary for secretion [15].

Recombinant Glycoproteins from CHO Mutant Cell Lines

Many CHO glycosylarion mutant cell lines with mutations in glucosidases, mannosidases, GlucNAc transferases, galactosyltransferases and sialyltransferases have been produced which give predominantly premature oligosaccharide intermediates [14]). Expression of recombinant glycoproteins in CHO glycosylation mutant cell lines called CHO-gmt cells produce recombinant glycoproteins with consistent structures [14,16]. However the CHO-gmt cells still do not resolve the issue of N-glycosylation sequon site variable occupancy since glycosylation heterogeneity was observed in the expression of rEPO [14]. Expression of human recombinant EPO in CHO wild type and CHO mutant line termed RCA-1 resistant mutant gave 25-37kd smear and a 23-25kd smear respectively for human recombinant EPO [14]. For therapeutic applications where tertiary oligosacchardies are required RCA-1 CHO mutant cell line which has a mutation in the N-acetylglucosaminyl transferase I giving only Man5GlcNAc2 oligosaccahrides is impractical.

Humanized Oligosaccharides from CHO Cells

Glycoproteins derived from murine NSO cells have > 50% the sialic acid variant – N-glycolylneuraminic which is immunogenic in humans [17]. CHO cells produce glycoproteins with α2,3 terminal sialic acid residues which are non-immunogenic though α2,6 sialic acid is preferred. CHO cells co-transfected with gamma-interferon (IFNγ) and α2,6 sialyl transferase produced sialylated IFNγ with 40% α2,6 sialic acid [18]. IFNγ with α2,6 sialic acid showed improved pharmokinetics than IFNγ from CHO cells without the α2,6 sialyl transferase co-transfected [18].

IgG antibody without fucose can be produced from CHO cells in which the fucosyl transferase has been knocked out. Recombinant IgG without fucose have enhanced attachment to Fc receptors with an increase in antibody-dependent-cell-mediated cytotoxicity (ADCC) and can be used to lyse cancer cells [19,20]. This is useful in the design of antibody therapeutics such as Hercptin an IgG antibody used to treat breast cancer where enhancement of ADCC ensures lower effective dosages [20].

GLYCOSYLATION HETEROGENEITY OF RECOMBINANT GLYCOPROTEINS FROM CHO CELLS

Mutagenesis to Eliminate Variable N-Glycosylation site Occupancy

Expression of secreted Thy-1 with three N-glycosylation sites at Asn 23, 74 and 98 from COS-7 cells showed variable site occupancy [12,13]. When secreted Thy-1 was expressed with Asn 23, 98 intact and the Asn 74 mutated to Serine it did not show variable site occupancy while with Asn 74, 98 showed variable site occupancy [13]. Similarly secreted Thy-1 with only Asn 23 or only Asn98 did not show variable site occupancy [13].

Lowered Rate of Protein Translation – for Complete N-Glycosylation Site Occupancy

The ER membrane bound oligosaccharyl transferase (OST) enzyme which transfers the dolichol oligosaccharide precursor to the N-glycosylation site of the glycopreptide is dependent on the protein translation rate (21). It has been shown that the glycan site occupancy in recombinant prolactin is dependent upon the rate of the elongation step of protein synthesis [22]. C127 mouse cells expressing recombinant prolactin had increased glycosylation efficiency from 20% to 80% in the presence of cycloheximide an inihibitor of protein elongation [22]. Cycloheximide extends therefore the time available for occupancy of the N-glycosylation site by reducing the elongation rate [22]. Recombinant human tPA from CHO cells shows variable site occupancy. N-glycosylation site occupancy improves at a reduced rate of protein translation such as by using butyrate in the media or lowering the culture temperature or by varying the fraction of cells in G0/G1 phase [23]. Lowering of the rate of protein elongation increases the exposure time of the glycan site to the oligosaccharide transferase enzyme in the ER (23). The residence time of proteins in the Golgi determines the processing by golgi resident glycosyltransferase enzymes. Cells incubated at 21^{o}C slowed the flow of glycoproteins through the golgi had 100% more N-acetyllactosamine [23]. Similarly polylactosamine extension in lysosomal membrane glycoprotein (LAMP-2) was dependent on the Golgi residence time [24].

GPI ANCHORED RECOMBINANT GLYCOPROTEINS - COMPLETE N-GLYCOSYLATION OCCUPANCY

GPI Anchored Membrane Bound vs Secreted Recombinant Thy-1

Secreted recombinant Thy-1 which has 3 N-glycosylation sites from CHO-K1 cells had a heterogenous molecular weight of 14-28kd with variable N-glycosylation site occupancy (Figure 2a, lane 1; 11). However, recombinant native GPI

anchored Thy-1 from CHO-K1 cells does not show variable site occupancy in that the completely glycosylated Thy-1 was obtained at 28kd by SDS-PAGE and western blot (Figure 2a, lane 2; 11). Standard protein discontinuous SDS-PAGE followed by western blot is the only visual technique to show variable site occupancy of a given protein. The expression of completely glycosylated glycoprotein without variable site occupancy indicates; (i) the attachment of the protein to the cell membrane ensures completet processing by Golgi membrane bound oligosaccharide processing enzymes resulting in a homogenous glycoprotein population and/or; (ii) glycoforms or unglycosylated protein are efficiently degraded by the cells quality control mechanism leading to the exclusive expression of the fully glycosylated glycoprotein.

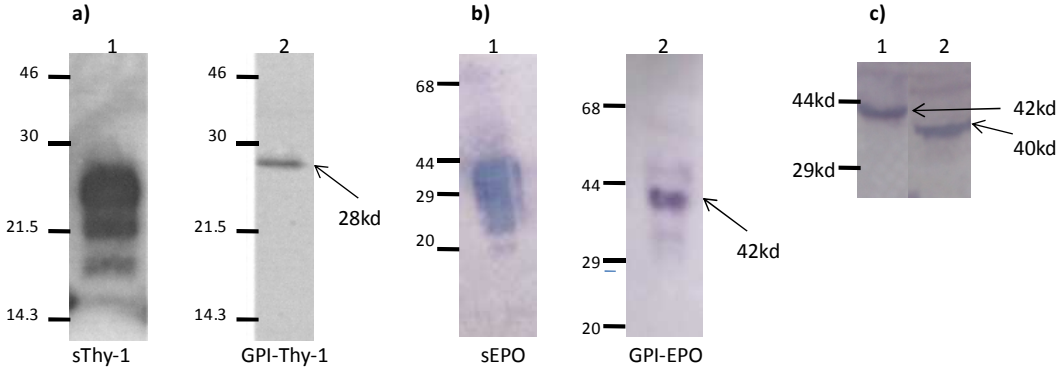

Figure 2: Comparison of Recombinant a) Secreted Thy-1 (Lane 1) and GPI Anchored Thy-1 (Lane 2); b) Secreted rEPO (Lane 1) and GPI Anchored rEPO (Lane 2); c) rEPO-GPI Anchored (Lane 1) and rEPO-GPI Anchored after Thrombin Digestion to Remove the GPI Anchor

Expression of Secreted Human Recombinant EPO

Recombinant EPO (rEPO) expressed in CHO cells is used in the treatment of anaemia. rEPO is a 166 amino acid glycoprotein with one serine O-linked oligosaccharide (ser126) and 3 N-linked glycosylation sites (Asn 24,38 and 83). EPO with biantennary oligosaacharides has low *in vivo* biologic activity due to rapid clearance from the systemic circulation by renal handling [25]. EPO with tertiary branched oligosaccharides is suggested to have a higher plasma level with an effective transfer to target organs and stimulation of erythroid progenitor cells [25]. CHO cells derived secreted human rEPO is fully active *in vivo* [26]. rEPO expressed in CHO cells demonstrates glycosylation heterogeneity with molecular weight of 34-38kd or 34-45kd by western blot [27,28].

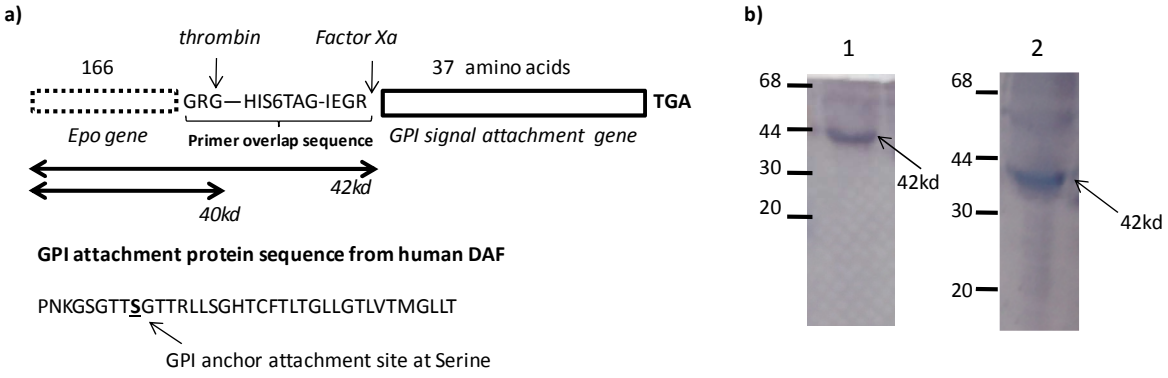

Figure 3: a) Schematic Representation of rEPO- GPI Anchored with the GPI Signal Sequence from DAF Shown; b) Comparison of rEPO GPI Anchored from CHO Adherent Cells in LM-15 Media (lane 1) and from CHO Cells in Suspension using Serum Free Media (Lane 2)

Expression of Human Recombinant EPO as a GPI Anchored Protein

Completely glycosylated human rEPO was obtained when expressed as a GPI anchored molecule in CHO cells with the GPI anchor signal sequence from decay accelerating factor [29] (Figure 3a). Homogenous completely glycosylated human rEPO-GPI anchored at 42kd without low molecular weight glycoform variants was obtained (Figure 2b; lane 2) as compared to heterogenous secreted rEPO from CHO cells (Figure 2b; lane 1) [29, 30]. The removal of the GPI anchor was designed to include a thrombin cleavage site C terminal to the rEPO gene (Figure 3a) [29]. The 1.8kd remaining 9 amino acid linker region between the rEPO and GPI anchor was removed using thrombin to give fully glycosylated human rEPO at 40kd with an extra GR dipeptide at the c-terminal (Figure 2c; lane 1 and lane 2) [30]. This is the first report for the production of a homogenous and completely glycosylated human rEPO at 40kd from CHO cells relevant for efficient therapy [30]. This completely glycosylated rEPO with possibly tetraantennary N-oligosaccharides, since of a higher molecular weight similar to urinary human rEPO will not be filtered from the kidney. Therefore rEPO will be maintained at a high plasma level resulting in the efficient stimulation of erythroid progenitor cells [25].

Human rEPO-GPI Anchored From Suspension Cells

For large scale purification of rEPO-gpi protein cells were grown as a suspension culture in serum free media (Hi media, Mumbai, India). The rEPO-gpi expressed by CHO suspension cells (Figure 3b; lane 2) (unpublished result) was no different from CHO adherent cells (Figure 3b; lane 1) at 42kd with complete N-glycosylation sequon occupancy and no glycosylation heterogeneity (Figure 3b; lane 2) (unpublished result). This was unlike that seen for human recombinant INF-γ from CHO as suspension cells where the proportion of unglycosylated IFN-γ was found to increase compared to from adherent CHO cells [31].

CONCLUSIONS

N-glycosylation occurs co-translationally in the ER where the oligosaccharyl transferase (OST) enzyme an ER memebrane bound enzyme transfers the dolichol oligosaccharide precursor to the N-glycosylation site of the glycopeptide [21]. The oligosaccharyl transfer reaction is dependent on the protein translation rate [21]. This is perhaps the reason for total N-glycosylation site occupancy of rEPO-GPI since the GPI anchor slows the transfer of the rEPO-GPI from the ER membrane to the golgi and allows for total Nglycosylation site occupancy by the ER bound oligosaccharyl transferase enzyme [29]. Completely glycosylated recombinant rEPO was obtained from CHO cells by the addition of a DAF GPI anchor signal sequence C terminal of the recombinant glycoprotein.

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